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Invertebrate Iridovirus Modulation of Apoptosis*

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Abstract: Programmed cell death (apoptosis) is a key host response to virus infection. Viruses that can modulate host apoptotic responses are likely to gain important opportunities for transmission. Here we review recent studies that demonstrate that particles of Invertebrate iridescent virus 6 (IIV-6) (*Iridoviridae*, genus *Iridovirus*), or an IIV-6 virion protein extract, are capable of inducing apoptosis in lepidopteran and coleopteran cells, at concentrations 1000-fold lower than that required to shut-off host macromolecular synthesis. Induction of apoptosis depends on endocytosis of one or more heat-sensitive virion component(s). Studies with a JNK inhibitor (SP600125) indicated that the JNK signaling pathway is significantly involved in apoptosis in IIV-6 infections of *Choristoneura fumiferana* cells. The genome of IIV-6 codes for an inhibitor of apoptosis *iap* gene (193R) that encodes a protein of 208 aa with 15% identity and 28% similarity in its amino acid sequence to IAP-3 from *Cydia pomonella* ganulovirus (CpGV). Transcription of IIV-6 *iap* did not require prior DNA or protein synthesis, indicating that it is an immediate-early class gene. Transient expression and gene knockdown studies have confirmed the functional nature of the IIV-6 *iap* gene. We present a tentative model for IIV-6 induction and inhibition of apoptosis in insect cells and discuss the potential applications of these findings in insect pest control.

Key words: Apoptosis; Invertebrate iridescent virus 6; Induction by endocytosis; Inhibition of apoptosis (iap) gene; *Iridoviridae*; JNK pathway involvement.

The family *Iridoviridae* comprises large icosahedral viruses with an internal lipid membrane and a large dsDNA genome (~100-210 kb) that is circularly permuted and terminally redundant (8). The family currently comprises five genera, of which two, *Iridovirus* and *Chloriridovirus*, encompass viruses that have been isolated solely from invertebrates and are collectively known as invertebrate iridescent viruses (IIVs).

IIVs infect >100 species of invertebrates, the majority of which are insects and terrestrial isopods, that usually occupy damp or aquatic habitats during at least one stage of the host life cycle (65). The range of insect species naturally infected by IIVs includes

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medically-important species of mosquitoes, midges and blackflies and agricultural pests, including several lepidopteran species of great economic significance. IIVs particles assemble in the cytoplasm and are arranged in paracrystaline arrays that result in infected hosts taking on a distinct iridescent appearance. The iridescent hues are the characteristic sign of patent disease that is invariably lethal to the host and from which this family of viruses gets its name.

To date, IIVs have attracted little interest due to the often low prevalence of patent disease in host populations. However, unapparent infections, that do not kill the host, may be orders of magnitude more common than patent infections by these viruses (44, 62, 64). Moreover, sublethally infected insects can show marked reductions in their reproductive capacity, adult longevity and body size, at least in the case of the mosquito *Aedes aegypti* (45). The question of how IIVs transmit between hosts remains largely unanswered, although predation or cannibalism of patently infected individuals involving the consumption of massive quantities of virus particles was demonstrated as a viable route of transmission in terrestrial isopods, crickets and one species of Lepidoptera (18, 19, 24, 67). Alternative routes exploit the high infectivity of IIVs by injection, for example, nematode or parasitoid-mediated transmission can be highly efficient and involve the introduction of small numbers of particles into host tissues during host penetration or wasp oviposition, respectively (43, 50).

The type species of the *Iridovirus* genus is *Invertebrate iridescent virus 6* (IIV-6), also known as Chilo iridescent virus, which was originally isolated from the rice stem borer *Chilo suppressalis* (Lepidoptera: Crambidae) in Japan (20). IIV-6 is one of only two IIVs to have been completely sequenced to date (14, 34). The genome is 212,482 bp, with a G+C content of 29%, and encodes 193 non-overlapping ORFs, or 234 ORFs if overlapping ORFs are included (34). Strains of this virus have been identified infecting commercial colonies of crickets in northern Europe (36, 38), and the fall armyworm in Mexico (67). The range of species that can be infected with this virus by injection is extensive and includes numerous species of medical and agricultural importance (21, 26, 35, 49, 53), in addition to a number of non-insect arthropods (54). Similarly, IIV-6 is capable of replicating in a wide range of cell lines from insects (1, 12, 15, 22, 31), and even a reptile (46) at temperatures of <30℃, whereas mammalian cell lines are not permissive (37).

The ability to effectively manipulate host cell defenses is necessary for virus survival and provides immediate rewards in generating opportunities for the transmission of progeny virus particles. The best characterized and most important of host cell responses to viral infection is apoptosis, a programmed sequence of events leading to cell death. We review recent advances that have provided new insights into the mechanisms by which IIVs elicit and inhibit host apoptotic responses. We also highlight novel avenues of research that offer possible biotechnological applications for the components of the IIV system for apoptosis control.

A BRIEF OVERVIEW OF APOPTOSIS

Apoptosis is an energetically-active, orchestrated sequence of biochemical processes, characterized by a series of distinct morphological changes that lead to cell death. This process is of key importance in development, cell number homeostasis, the eradication of

damaged or defective cells and in the control of infection (42). The main steps in apoptosis involve (i) chromatin condensation and endonuclease-mediated degradation of nuclear DNA, (ii) disruption of the cytoskeleton leading to cytoplasmic shrinkage and detachment from neighboring cells, (iii) nuclear fragmentation and cell blebbing that results in the appearance of apoptotic bodies, and finally (iv) phagocytosis of apoptotic bodies that exhibit phosphatidlyserine on the outer membrane, and subsequent degradation by the lysosomes of phagocytic cells. Importantly, cell contents are repackaged during apoptotic fragmentation without leakage of the cells' constituent components.

There are two major pathways by which cells can be triggered to undergo apoptosis. The extrinsic pathway, which relies on signals from outside of the cell, involves activation of the tumor necrosis factor (TNF) or Fas-ligand receptors that bind to death domain associated proteins (TRADD/FADD) that together form the Death-inducing signal complex (DISC) that directly activates caspase-8, an apical or initiator caspase. Caspases are cysteine proteases that function as homodimers and which are activated by cleavage. Caspase-8 activates effector caspases (caspase-3, -6, -7) that are responsible for triggering the cascade of apoptotic events. Apoptosis in insects is best understood in the fruitfly *Drosophila melanogaster* (Diptera: Drosophilidae) (25). In *Drosophila*, one mechanism includes the interaction of Eiger (invertebrate TNF ligand) with the wengen membrane receptor. This leads to activation of c-Jun N-terminal kinase (JNK), which then triggers the central initiator caspase Dronc, instigating a cascade that sets off the effector caspase Drice and ultimately cell apoptosis. As such, the Eiger-wengen mediated pathway appears to be the invertebrate equivalent of the extrinsic pathway found in mammalian systems (25).

In contrast, the intrinsic pathway relies on signals from the mitochondria in response to cellular stress, toxins or UV damage. Permeabilization of the mitochondrial membrane results in loss of mitochondrial transmembrane potential, a halt to mitochondrial bioenergetic functions, and the release of mitochondrial intermembrane space proteins into the cytosol. These proteins include caspase activators like cytochrome c and Smac/Diablo, as well as caspase-independent death effectors including an apoptosis-inducing factor (AIF) and endonuclease G. Cytochrome c binds to Apaf-1 (Apoptotic protease activating factor 1) and these complexes undergo a dramatic assembly process to form a disk-shaped heptamer, known as the apoptosome, which serves as an activation machine for initiator caspase-9 and effector caspases, such as capsase-3. However, in insects the role of mitochondrial pathways during apoptosis induction remains unclear (52). Induction of apoptosis in uninfected *Spodoptera frugiperda* (Lepidoptera: Noctuidae) cells appears to involve the equivalents of mammalian apical caspase and caspase-3 as initiator and effector caspases, respectively (25), whereas in *Drosophila*, the Dronc caspase may be activated through endoplasmic reticulum stress or through Bcl-2-type stimulation (discussed below), and these could represent invertebrate equivalents of the intrinsic pathways described from mammals.

As would be expected, a number of cellular anti-apoptotic factors regulate the pro-apoptotic factors and the decision to initiate apoptosis is determined by the balance between the cells own promoting and inhibiting

entities. For the extrinsic pathway the cellular TNF Receptor Associated Factors (TRAFs) activate MAP kinase that in turn activates IκB kinase (IKK) which liberates NF-κB from its inhibitory complex leading to NF-κB-mediated upregulation of antiapoptotic genes, such as TRAFs that prevent DISC complex formation, or FLICE-like inhibitory protein (cFLIP) that inhibits caspase-8 activation.

For the intrinsic pathway the principal inhibitory agent is Bcl-2, which is located on the external mitochondrial membrane and which blocks apoptosis by binding to Apaf-1 thus preventing activation of capsase-9. Bcl-2 also stabilizes the permeability of the mitochondrial membrane (23). The antiapoptotic Bcl-2 and the closely related Bcl-xL each possess four BH domains whereas related members of the Bcl-2 family, notably Bid, Bax, Bak and Bad, the so-called BH3 only proteins, only possess BH domain 3 and are proapoptotic in nature. Bid interacts with Bax and/or Bak to form an oligomeric pore in the outer mitochondrial membrane whereas Bad forms a heterodimer with Bcl-2/Bcl-xL thereby eliminating the inhibitory effect on Apaf-1 and allowing Bax/Bak-triggered apoptosis.

The final group of antiapoptotic entities is comprised of the cellular Inhibitors of apoptosis (cIAP) which have the potential to inhibit apoptosis activated by either the intrinsic or the extrinsic pathways. This is because they can bind to both activator and effector caspases and appear to be present ubiquitously in metazoan organisms. IAPs were first identified in a baculovirus (13) and are characterized by one, but usually two or three, baculoviral IAP repeat (BIR) domains, consisting of approximately 70 amino acids, and a zinc-binding C-terminal RING finger domain (48). cIAPs are themselves negatively regulated by the mitochondrial proteins Smac/Diablo and Omi/HtrA2, the latter being a serine protease that binds to, and cleaves, cIAP. The mode of action of IAPs has been best characterized for the mammalian X-linked IAP (XIAP) which acts by binding at BIR domain 3 (BIR3) to caspase-9 at a site that prevents caspase dimerization, forcing the caspase active site to adopt an inactive conformation (58). The interaction of XIAP with effector caspases is quite different and involves pairs of dimerized caspases. For caspases-3 or -7 the linker region preceding the BIR2 domain blocks the catalytic site of one caspase dimer whereas the IAP binding motif from the opposing dimer binds to a groove on BIR2 (68). In contrast, in insect cells, it appears that baculovirus IAPs may function by blocking the activation of procaspases, such as procaspase-1 (57). Finally, IAPs may have an additional means of exerting their effects by functioning as E3 ubiquitin ligases. In *Drosophila*, the RING domain of a cIAP (DIAP1) can recruit a ubiquitin conjugating enzyme (UBC) to an initiator capsase (Dronc) or other pro-apoptotic proteins which, once ubiquitinated, are flagged for proteasome-mediated degradation or modification (63). The mode of action of baculovirus IAPs is notably different from that of other baculovirus antiapoptotic proteins such as P35 or P49 that block virus-induced apoptosis downstream of the effector caspase Drice in *Drosophila* cells (40).

We cannot finish this section without mentioning the role of microRNAs (miRNAs) in post-transcriptional regulation of gene expression, a topic that is currently attracting enormous interest. The role of miRNAs in regulating apoptosis is still far from clear although the *miR-2* family of miRNAs has been

identified as regulators of *reaper, grim* and *sickle* proapoptotic genes in *Drosophila* cells (60), whereas the proapoptotic *hid* gene is targeted by the microRNA *bantam* (4). Indeed, it seems that miRNA modulated processes are likely to prove highly influential in regulating pathogen induced apoptosis in *Drosophila* (5) and across a wide range of other taxa (11).

INDUCTION OF APOPTOSIS BY IIV-6

The mechanisms by which iridoviruses gain entry to the cell, replicate and transmit themselves has been described elsewhere in this special issue of *Virologica Sinica* (51) and in recent reviews by others (9, 66). Recently, Bilimoria and colleagues showed that a virion protein extract (VPE) prepared from IIV-6 particles induced apoptosis in spruce budworm (IPRI-CF124T) and boll weevil (BRL-AG-3A) cell lines as detected by blebbing, DNA fragmentation, and TUNEL assay (55). Tissue culture assays revealed that spruce budworm cells were eight times more sensitive to VPE than boll weevil cells. A pancaspase inhibitor suppressed apoptosis but its effect on the inhibition of host protein synthesis was marginal. Furthermore, the effective VPE dose for induction of apoptosis was 1000-fold lower than that required for shutdown of host protein synthesis. Protein kinase activity was also detected in VPE. Heating VPE at 60℃ for 30 min destroyed all three activities, suggesting that one or more polypeptides in the IIV-6 particle are responsible for inducing apoptosis.

To determine whether exterior or interior stimuli were required for IIV-6 induced apoptosis a series of experiments was performed using polystyrene-bound virus particles and chemical endocytosis inhibitors. The interaction of IIV-6 particles with the cell surface failed to induce apoptosis in studies in which cells were treated with UV-inactivated virus that was first bound to polystyrene beads. Moreover endocytosis inhibitors (bafilomycin or ammonium chloride) blocked the induction of apoptosis by intact or UV-inactivated virus, or VPE, indicating that entry of one or more virion components via endocytosis is required for induction of apoptosis. Furthermore, the strong apoptotic response to VPE compared to the weak response observed following treatment with infectious IIV-6 particles led these authors to postulate that viral gene expression may have been involved in inhibiting host apoptosis (10).

INHIBITION OF APOPTOSIS BY IIV-6

Chitnis *et al*. (10) were the first to demonstrate JNK signal requirement during apoptosis induction by an insect virus. High concentrations of intact IIV-6 particles (400 μg/mL), UV-irradiated virus (10 μg/mL) and VPE (10 μg/mL) induced apoptosis in 60% of treated lepidopteran cells (*Choristoneura fumiferana*; IPRI-CF-124T), whereas a moderate concentration of infectious IIV-6 (10 μg/mL) induced apoptosis in only 10% of cells. Apoptosis was inhibited by the apical caspase inhibitor, Z-IETD-FMK, indicating that caspase activity is required for virus-induced apoptosis. Further studies revealed that treatment with UV-irradiated virus or VPE enhanced virus-induced caspase activity by 80%, relative to mock-treated cells, at 24 h after treatment. As the MAP kinase pathway may induce or inhibit apoptosis depending on cellular context a JNK inhibitor (SP600125) was used to demonstrate drastic suppression of VPE-induced apoptosis. It appears, therefore, that the JNK signaling pathway is signifycantly involved in apoptosis in IIV-6 infections of *C. fumiferana* cells.

The weak apoptotic response to moderate concentrations of infectious virus particles led these workers to postulate that viral gene expression may be involved in inhibiting apoptosis (10). Infection of cells in which protein synthesis was inhibited with cycloheximide resulted in 69% of the cell population undergoing apoptosis compared to 10% in normal infection indicating that *de novo* synthesis of viral proteins was not required for viral induction of apoptosis. Furthermore, blocking viral DNA replication with aphidicolin or phosphonoacetic acid suppressed apoptosis and virus-induced caspase activity, suggesting that early expression of viral genes was necessary for inhibition of apoptosis. These results were quickly confirmed by Ince *et al* (33) in studies on a candidate inhibitor of apoptosis gene in IIV-6.

The genome of IIV-6 codes for an *iap* gene (193R), first identified by Birnbaum *et al*. (3) as having one BIR domain and a C3HC4 RING finger motif at the C-terminal end of the ORF. The ORF 193R is 624 bp (208 aa) in length with a predicted mass of 22.8 kDa, and shares 15% identity and 28% similarity in its amino acid sequence to IAP-3 from *Cydia pomonella* ganulovirus (CpGV), the first IAP protein identified in baculoviruses (13). Recently, this ORF was conclusively demonstrated to be a functional *iap* gene (33). Transcription studies revealed that IIV-6 *iap* transcripts were detectable from 4 h post-infection and that transcription did not require prior DNA or protein synthesis, indicating that IIV-6 *iap* is an immediate early class gene. Transient expression of IIV-6 iap under the control of the AcMNPV *ie1* promoter prevented cellular DNA fragmentation and was as effective as transiently expressed AcMNPV *p35* in reducing actinomycin-D induced apoptosis of two insect cell lines. Finally, permissive *Bombyx mori* cells (SPC-BM-36) inoculated with a high concentration of IIV-6 particles developed apoptotic like vesicles at 24 h post-infection, but these structures disappeared later in infection and no evidence of genomic DNA fragmentation was observed in virus-treated cells. In contrast, cells in which the *iap* gene was knocked down by dsRNA transfection underwent prolific apoptotic body formation and DNA fragmentation at 24-48 h post-infection (33).

APOPTOSIS IN VERTEBRATE IRIDOVIRUS HOSTS

Induction of apoptosis has been reported in a number of fish and amphibian iridoviruses (28, 30, 32, 39). Notably, untreated, heat-inactivated or UV-inactivated Frog virus 3 (FV-3, genus *Ranavirus*) all triggered apoptosis of fish and mammalian cells, indicating that intact viral DNA or viral polypeptides were not required to initiate apoptosis (6). Not surprisingly, Epizootic hematopoietic necrosis virus (EHNV) induced apoptosis of fish epithelioma cells was markedly reduced in the presence of a nonspecific protein kinase inhibitor (17). A number of putative antiapoptotic genes have been identified in the vertebrate iridoviruses including a Bcl-2 protein, ubiquitin ligases with RING finger domains, and a SAP domain containing peptide (59) as well as TNF-α, TNF receptor, Cathepsin-B and CARD-like caspase orthologs which may have potent proapoptotic properties (16, 29, 59, 61). However, to date, only the Grouper iridovirus (GIV) Bcl-2 homolog has been demonstrated to be a functional antiapoptotic gene (41). The GIV-Bcl protein is also present in the virion.

SIGNIFICANCE OF APOPTOSIS FOR IIV-6

All this leads us to present a working model for the biological significance of apoptotic and host shutoff effects of IIV-6 (Fig. 1). The active polypeptide(s) present in infecting virus particles inhibit host protein synthesis regardless of viral gene expression. This allows the virus to take control of the cellular synthetic machinery. The host responds by initiating apoptosis and the virus in turn, expresses an immediate early gene (*iap 193R*) whose product inhibits apoptosis (33). This block on apoptosis is so effective that sufficient time is available for massive numbers of virus particles to be assembled in each cell prior to lysis and virus dissemination.

According to the *Drosophila*-based model (Fig. 1), the virion component (s) of IIV-6 activates JNK, which results in reaper-induced apoptosis via the Dronc initiator caspase. Reaper inhibits protein synthesis. Consequently, Chitnis *et al*. (10) have postulated that JNK is upstream of reaper and the Dronc-like virus-induced caspase in *C. fumiferana* cells. If this model is valid, then a JNK inhibitor should block translational inhibition. However, if the model does not hold, then phosphorylation of eukaryotic initiation factor eIF2α must also be investigated. In *Drosophila,* an endoplasmic reticulum-resident kinase (Dperk) phosphorylates eIF2α in response to stress (56). Infection by the vertebrate iridovirus FV-3 results in phosphorylation of host eIF2α and translational shut-off (7). If a component of the IIV-6 virion is capable of phosphorylating eIF2α, we would expect translation to be similarly inhibited. For the time being, this model awaits empirical testing and validation.

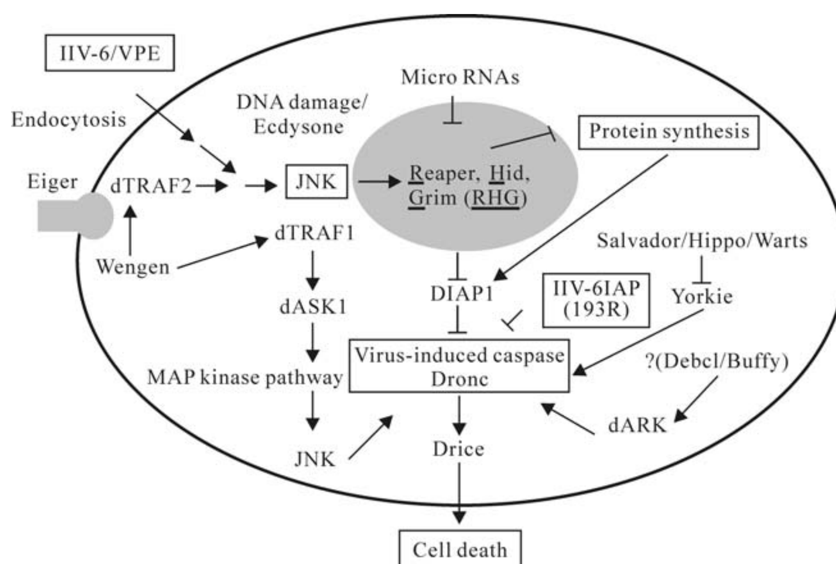

Fig. 1. Working model for induction and inhibition of apoptosis by IIV-6 in insect cells based on a *Drosophila* cellular system. The proposed pathway is shown in black, other cellular pathways are shown in grey. Virus particles or components of the virion protein extract (VPE) enter the cell via endocytosis. Kinase activity in particles/VPE phosphorylates JNK or another member of the MAP kinase pathway upstream of JNK. JNK then activates RHG proteins, such as Reaper, that dissociates the cellular inhibitor of apoptosis DIAP1 from Dronc leading to degradation of DIAP1. Reaper also inhibits host protein synthesis, which may in turn enhance apoptosis by reducing cellular levels of DIAP1 (dashed line) (27). Activated Dronc and/or a virus-induced apical caspase then activates the effector caspase Drice which then carries out destruction of the cell. Early expression of the IIV-6 *iap* gene (ORF 193R) inhibits Dronc/virus-induced caspase thus avoiding activation of Drice.

FUTURE DIRECTIONS

Cotton boll weevil, *A. grandis* (Coleoptera: Curculionidae) larvae treated with intact particles of IIV-6 or VPE under laboratory conditions suffered mortality and metamorphic arrest (2, 47). Similarly, reduced population survival has been observed in the case of the cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) following treatment with VPE (2). These findings prompt the question of the degree to which virus-induced apoptosis, inhibition of host protein synthesis and mortality/metamorphic arrest are interrelated; an issue that merits future study. Although it is possible that these viral entities may be used directly to control insects, identification of the active polypeptide is currently under investigation (S.L. Bilimoria, unpublished data). The genes for active polypeptides may then be cloned into vectors for transformation of plants or plant colonizing microorganisms, thereby providing a novel method for controlling insect infestations. In addition to explicitly testing the apoptotic model described above, complimentary studies involving identification of the polypeptides that are primarily responsible for triggering or inhibiting apoptosis would also facilitate research into the mechanisms by which the virus subverts cellular functions for its own survival with diverse potential applications in biotechnological advances and understanding the molecular basis for viral pathogenesis in insects.

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